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Cryptands and related tripodal ligands: interaction with nucleic acids and nuclease activity of their Eu(III) complexes

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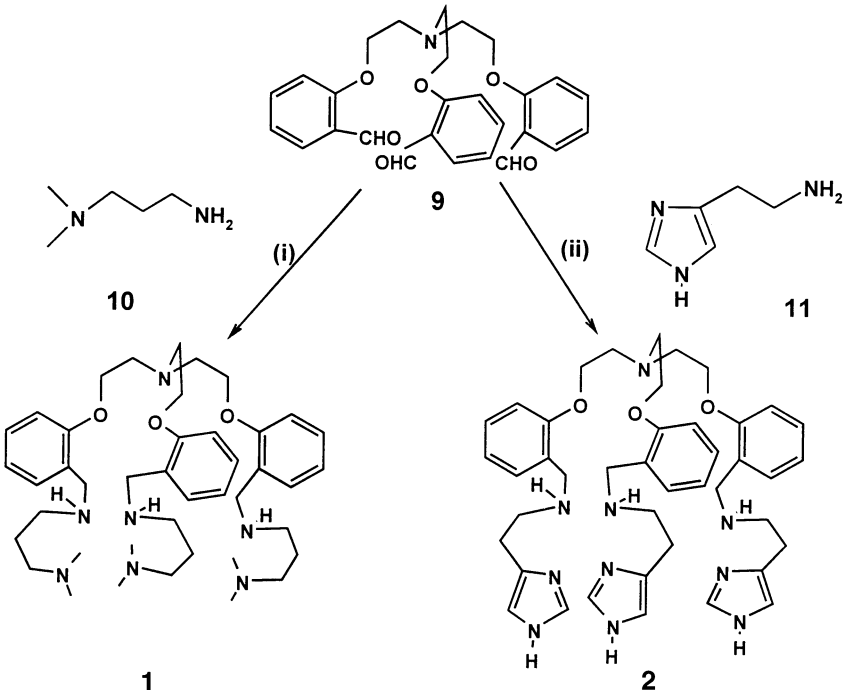
Abstract—Three tripodal ligands (**1**–**3**), three macrobicyclic cryptands (**4**–**5**), the [222] and [221] cryptands **7** and **8**, and corresponding Eu(III) complexes were probed for their influence on double-stranded nucleic acids. One ligand (**2**) shows higher affinity to DNA as compared to RNA and another one (**4**) even slightly destabilizes RNA. Cryptands **7** and **8** show no detectable melting point effects; addition of the [222] cryptand **7** to Eu(III) salts has no significant effect on the nuclease activity of the free metal ion. Only the [221] cryptand **8** forms a stable complex with Eu(III) in water, leading to almost no rate decrease with the phosphodiester BNPP, in striking contrast to DNA. The histidine-containing ligand **2** exhibits an increased hydrolytic activity against BNPP and against plasmid DNA.

1. Introduction

The interaction of nucleic acids with cryptands is of interest in view of their size and relative rigidity, as both factors can lead to different binding modes and affinities compared to acyclic polyamines.[1] In addition, cryptands could offer ways to provide even kinetic stabilities for chemical nucleases derived from such ligands and transition metal and/or



Scheme 1. Synthetic pathways. Reaction (i) with *N,N*-dimethylpropylenediamine followed by reduction; reaction (ii) with histamine followed by reduction.

Keywords: polyamines; cryptands; europium complexes; DNA/RNA affinities; phosphorester cleavage; catalysis.
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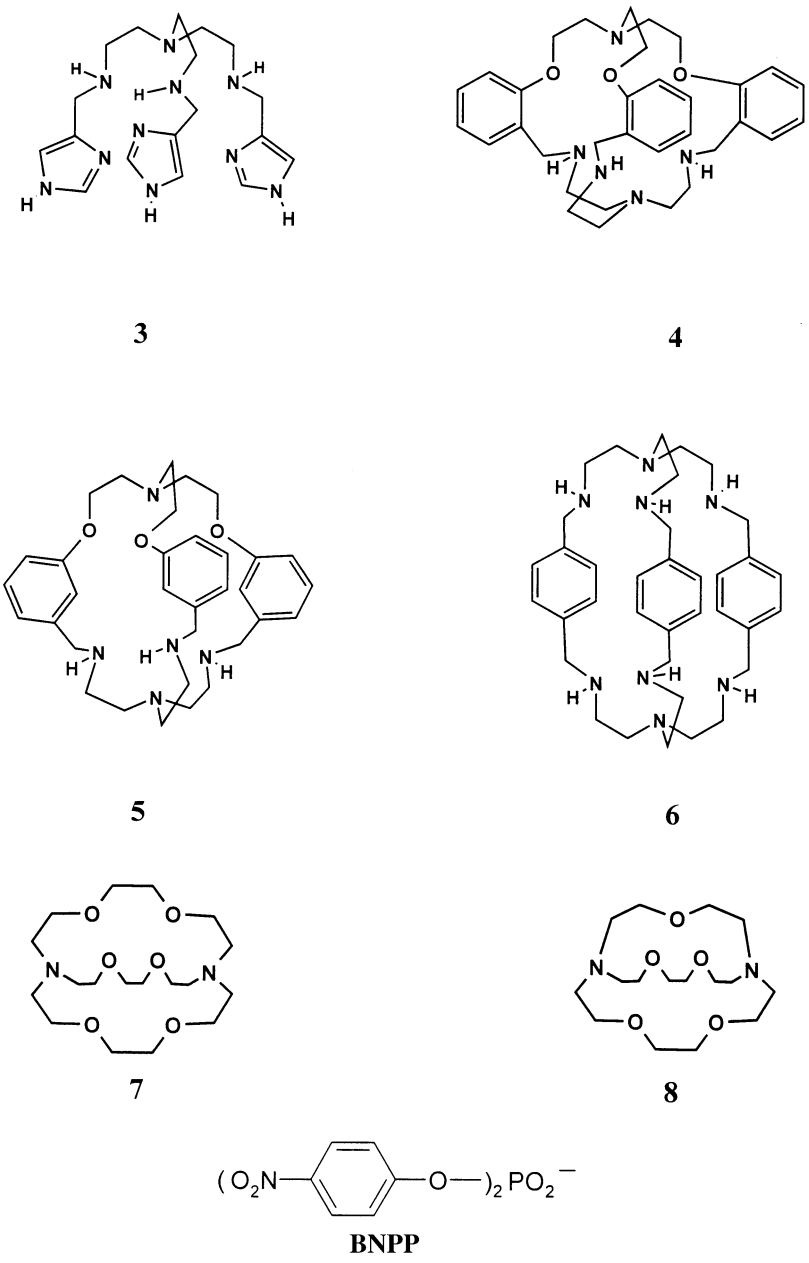

Scheme 2. Structures of ligands.

lanthanide cations.[2] If the catalytic activity is not hampered in such complexes, they would offer a starting point for the attachment of rate-enhancing co-factors. It should be noted, that at least lanthanide complexes used so far for such a purpose have the disadvantage of either low stability, or they contain carboxylic groups which lower the nuclease activity,[3] with only few exceptions.[4] The [222] cryptand **7** in combination with e.g. Eu(III) ions was suggested early[3b,c] for such nucleases as a solid state structure showed an inner-sphere complex for this system.[5] However, a recent NMR investigation[6] established that in water no complexation inside the cavity occurs, in contrast to the crystalline state. These recent findings explain why the combination of the [222] cryptand **7** and Eu(III) salts showed no significant effects on the catalytic power of the metal ion, The smallest possible ligand in the form of the [221] cryptand **8** forms innersphere complex with Eu(III) which also shows dissociation in water, albeit with slower rate.[7] These results suggested the use of other cryptands with architectures designed to entrap a lanthanide ion inside the cavity while showing enhanced affinity towards DNA/RNA. The investigation of such systems, including the smallest possible ligand in the form of the [221] cryptand **8** hitherto not investigated, was therefore another aim of the present study besides the above mentioned affinity variations with nucleic acids.

2. Results and discussion

Synthesis of the ligands **1**, **2** and **3** are described below while **4**, **5** and **6** were prepared as described earlier[8] (Schemes 1 and 2). A tripodal trialdehyde **9** was prepared following the literature procedure.[8a] The ligand **1** was prepared by the

Table 1. Ligand interaction with polyA–polyU and polydA–polydT

Ligand	No. of positive charges	r[a]	ΔT_m in °C PolyA–polyU	ΔT_m in °C PolydA–polydT
1[b]	~6	0.1	28.2	11.3
		0.2	[c]	[c]
		0.3	[c]	[c]
2	~3.9	0.1	16.7	5.3 and 16.3
		0.2	19.4	22.7
		0.3	[c]	[c]
3	~3.0	0.1	23.9	4.3
		0.2	28.9	10.2
		0.3	32.2	13.2
4	~2.1	0.1	−0.5	1.0
		0.2	−1.8	2.1
		0.3	−4.0	2.3
5	~3.2	0.1	14.3	9.5
		0.2	13.0	14.2
		0.3	12.0	10.8
6	~6.0	0.1	6.3 and 29.8	20.0
		0.2	[c]	[c]
		0.3	[c]	[c]

Conditions: 0.01 M MES buffer; pH 6.25, I=0.01 M (adjusted with NaCl); error in ΔT_m=±0.5°C.
[a] r=molar ratio of ligand/nucleic acid phosphate.
[b] The cryptands [222] **7** and [221] **8** have no measurable effect on the T_m of polyA–polyU and polydA–polydT; at r=0.1, 0.2 and 0.3, the change was ΔT_m<1°C.
[c] Precipitation observed.

Schiff base condensation of this trialdehyde **9** with three equivalents of *N*,*N*′-dimethyl-1,3-diaminopropane **10** followed by in situ reduction with $NaBH_4$. Synthesis of **2** was achieved in a similar manner by replacing the amine with histamine base **11** but in a different solvent.

2.1. Affinity study by melting point experiments

The melting points of double-stranded nucleic acids indicate that linear and most macrocyclic polyamines show affinities toward both RNA and DNA with a preference for RNA.[9] The affinity generally increases with the average number of charge on the ligand structure.[1h] In most cases the affinity of macrocyclic amines is lower than that of the linear amines, with some exceptions.

The podands **1**, **2** and **3** show a lower affinity to both RNA and DNA than expected considering the number of positive charges, with a preference as usual for RNA with **1** and **3**. The number of charges in all ligands was estimated on the basis of known pK_a values.[10] For **1** at higher ratios of ligand to nucleic acid phosphate like r=0.2 and 0.3, precipitation is observed. The behavior of **2** is interesting as it binds preferably to DNA, in contrast to most other polyamines, or also **5** and **6** (Table 1).

With RNA the cryptand **4** shows even a destabilisation, with a very small stabilisation of DNA. Until now a related, although much stronger destabilisation was observed only with some special linear polyalkylamine derivatives containing non-intercalating phenylunits,[11] presumably due to stabilization of the unfolded or single stranded nucleic acid. In $CDCl_3$ solution also, π-stacking interactions are observed via NMR TOCSY experiments. In view of this, and in analogy to literature arguments[12] one may expect that the aromatic rings of **4** may preferably stack with exposed bases of unfolded nucleic acid parts and thus destabilize the double strands.[11]

The macrobicyclic compounds **4** and **5** both contain a tren (tris(2-aminoethyl)amine) **12** unit. The affinity of tren itself for RNA and DNA[9] may be compared with these ligands. While the affinity of **4** for both RNA and DNA is much lower than that of tren, the affinity of **5** is considerably lower than that of tren in the case of RNA, but interestingly comparable to tren in case of DNA. Ligand **6** has a preference for RNA, although to a lesser degree than **1**. In order to shed some light on the possible origin of the affinity differences, we estimated the distances between the positive charge centers in the ligands on the basis of force field optimizations (gas phase simulations with Hyperchem 6.0). Optimal matching between the cationic polyamine centers and the nucleic acid phosphate groups for a given amine differ due to the generally smaller and deeper RNA major groove as compared to the larger distances between phopshate anions in the major groove of DNA.The average distances d for different energy minima between the positive charge centers are for **1**: 5.0, 12.5, 16.5 and 20.5 Å; **2**: 11.5 Å; **3**: 6.4 Å; **4**: 5.6 Å; **5**: 6.5 Å and **6**: 5.7, 7.1 and 9.5 Å. Due to the high flexibility of the podands **1**–**3** little can be said about these ligands; the distances for the more rigid bicyclic compounds seem to match indeed. The average distance found in X-ray derived structures[13] between two positive charge centers in **4** is about 3.9 Å, while for **5** it is 4.2 Å. However, in these structures, a water molecule is included inside the cavity and is hydrogen-bonded to the amino nitrogens and one bridgehead nitrogen. That means, the water molecule may drag the amino nitrogens towards itself causing the distance between any two amino nitrogens to shrink.

2.2. Phosphoesterase activity

The phosphoesterase activity of some Eu(III)Cl_3 in presence of several ligands was probed by using BNPP (bis-(*p*-nitrophenyl)phosphate) as model substrate and with pBR322 plasmid DNA, using methods described before. Only the

Table 2. Pseudo-first order rate constants for BNPP and DNA hydrolysis with the Eu(III) complex of [221] **8**

Concentration $C\times10^3$ M	BNPP hydrolysis,[a] $k_{obs}\times10^4$ s^{-1}		k_{cat} and K_M for BNPP		DNA[b,c]
	[221]**8**$EuCl_3$	Only $EuCl_3$[d]	[221]**8**$EuCl_3$	Only $EuCl_3$[d]	
0.5	0.10	0.27	2.2×10^{-4} s^{-1}, 9.0×10^{-3} M	2.6×10^{-4} s^{-1}, 2.9×10^{-3} M	
1.0	0.24	0.70			
2.5	0.46	1.17			
5.0	0.79	1.67			
7.5	0.99	1.90			
10.0	1.15	1.92			

[a] Conditions: [BNPP]=3.76×10^{-5} M; 0.01 M EPPS buffer; pH 7.0; T=50°C.
[b] [DNA]=1.9×10^{-5} M (bp); 0.01 M EPPS buffer; pH 7.0; T=37°C; incubation time 2 h.
[c] No cleavage was detected for DNA hydrolysis with a concentration of 5×10^{-3} M of the complex.
[d] Addition of [222] cryptand leads to rate decrease with BNPP (see Ref. 4) and almost little effect with DNA (see Ref. 2g).

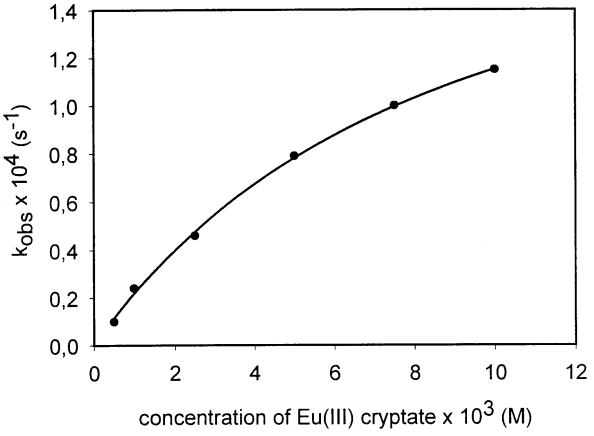

Figure 1. Saturation kinetics measured with Eu(III) complex of the cryptand [221] for the cleavage of BNPP.

[221] cryptand **8** forms a complex with sufficient stability in aqueous solution, as evident from NMR data. For BNPP the k_{obs} values found in presence of the [221] ligand **8** is lower than that of the metal ion alone. This lower activity may be explained by the decreased contact of the highly charged cation due to the encapsulation of the metal ion in the cavity of the cryptand. Analysis of the Michaelis–Menten like saturation kinetics (Table 2, Fig. 1) indicate that the k_{cat} value is close to that observed with free metal ion, but the K_M value is larger. For uncatalysed reaction k_{obs} value is 3×10^{-10} s^{-1}. Thus, at 10 mM concentration of the Eu(III) complex with [221] the rate enhancement is 3.8×10^5 times. For DNA hydrolysis, remarkably, there was no detectable cleavage with the Eu(III)/[221] **8** complex. This is in line with the unfavourable K_M values found already with BNPP: the encapsulated Eu(III) will have very low affinity with the DNA, at the same time the k_{cat} may be diminshed by less contacts. In addition, it is known[14] that the competing binding of free (protonated) amines alone as well as metal cations such as Na^+ at higher concentrations decrease the catalytic efficiency of lanthanide cations. Even the activity of Eu(III) against BNPP is decreasing in the presence of spermine[15] presumably again by blocking the phosphate interaction with the Eu(III) ion.

Table 3. Pseudo-first order rate constants for BNPP and DNA (% of RF I and RF II form for plasmid DNA cleavage) with Eu(III) complexes

Ligand	BNPP hydrolysis[a]		DNA hydrolysis[b]			
	$k_{obs}\times10^4$ s^{-1}	F[c]	%RF I	%RF II	$k_{obs}\times10^4$ s^{-1}	F[c]
1	0.54	0.77	64	36	0.62	1.05
2	1.26	1.8	25	75	1.92	3.25
4	0.48	0.68	[d]		–	–
5	0.49	0.70	[d]		–	–
No ligand[e]	0.70	1.0	65	35	0.59	1.00

[a] Conditions: [Ligand]=1×10^{-3} M; [$EuCl_3$]=1×10^{-3} M; [BNPP]=3.76×10^{-5} M; 0.01 M EPPS buffer (25% MeOH) r; pH 7.0; T=50°C.
[b] Conditions: [Ligand]=5×10^{-3} M; [$EuCl_3$]=5×10^{-4} M; [DNA]=1.9×10^{-5} M (bp); 0.01 M EPPS buffer (25% MeOH); pH 7.0; T=37°C; incubation time 2 h. Corrected for the decreased stainability of RF I by a factor of 1.22; corrected for the impurity of RF II in starting material and background noise; Double runs, error ±2.5%.
[c] Where $F=k_{obs}\times10^4/0.70$ for BNPP and $k_{obs}\times10^4/0.59$ for DNA.
[d] No bands could be located.
[e] Rates for $EuCl_3$ alone (Refs. 2g and 4).

Cleavage results of BNPP and plasmid DNA with the systems **1**–**5** in combination with Eu(III) are shown in Table 3. The ligands **4** and **5** may complex lanthanide ions in their cavities,[16] by binding with the three ether oxygens and three amine nitrogens of the chains. However, in spectrophotometric titration for the complexation of cryptand **4** with $PrCl_3$ in MeOH by monitoring the decrease of the extinction coefficient of Pr(III) ion at 444 nm, the log K value is found to be only 2.59. Complexation in water, which, due to solubility problems could not be measured, is expected to be much weaker. Therefore, this, as well as the other ligands cannot be expected to complex the Eu(III) ion in water to any significant degree. In consequence, these ligands can essentially either block the phosphate of the substrates, as discussed, with negative effects on the Eu catalytic power, or perhaps act as co-factors in the hydrolysis. This can be expected in particular from ligands **2** and **3** containg imidazole units, which were prepared also with this idea in mind. It has been shown earlier[17] that three histidine moieties are involved as co-factor in the phosphate ester hydrolysis by kbPAP (kidney bean purple acid phosphatase) enzyme. Addition of **2** leads indeed to a significant rate enhancement both with BNPP and with DNA. The observed accelerations are only moderate, but one has to bear in mind, that the usual negative effect of added polyamines (see above, Ref. 15) has to be overcome with all these ligands. The complexation constant of **2** with $PrCl_3$ is below the limit of measurement even in methanol as solvent (no saturation curve); it is so low in water that under conditions used for the kinetics almost all the lanthanide remains uncomplexed; at higher concentration

precipitation was observed. Considering this the very low complexation degree, **2** is remarkably active. The ligands **4** and **5** could not be located in the electrophoresis gel even after the treatment with an ion exchange resin which was found successful[2k] with other macrocyclic polyamine ligands.

3. Experimental

3.1. Materials

PolyA–polyU, polydA–polydT, BNPP, MES and EPPS were obtained from Sigma; other reagents were obtained from Aldrich; plasmid pBR322 DNA was purchased from Pharmacia. All solvents were purified prior to use.

3.1.1. Tris-{[2-(2-benzyl-*N*,*N*-dimethylpropylenediamine)-oxo]ethyl}amine (1). The starting trialdehyde[8a] (0.2307 g, 0.50 mmol) was dissolved in 10 mL dry MeOH by warming followed by addition of *N*,*N*′-dimethyl-1,3-diaminopropane (**10**) (0.1533 g, 1.50 mmol). The mixture was refluxed for 6 h, cooled down to room temperature and reduced in situ with excess (~ 5 mmol) of $NaBH_4$. The solvent was evaporated under reduced pressure, 10 mL of distilled water was added to the mixture, and the tripodal amine was extracted with 3×10 mL $CHCl_3$. The organic layer was dried over anhydrous Na_2SO_4, the solvent was evaporated under reduced pressure to obtain **1** as a semi-solid in 85% yield. (^{1}H NMR, $CDCl_3$, TMS, ppm) 1.63 (qt, –NHCH$_2$*CH*$_2$CH$_2$N(CH$_3$)$_2$, *J*=9.6 Hz), 2.15 (s, –N(*CH*$_3$)$_2$), 2.26 (t, –NHCH$_2$CH$_2$*CH*$_2$N(CH$_3$)$_2$, *J*=9.8 Hz), 2.58 (t, -NH*CH*$_2$CH$_2$CH$_2$N(CH$_3$)$_2$, *J*=9.6 Hz), 3.17 (t, –N*CH*$_2$CH$_2$O, *J*=7.4 Hz), 3.76 (s, –Ar*CH*$_2$NH–), 4.11 (t, –NCH$_2$*CH*$_2$O, *J*=7.5 Hz), 6.68 (m, *Ar*), 7.23 (m, *Ar*). Anal calcd for $C_{42}H_{69}N_7O_3$: C, 70.06; H, 9.66; N, 13.62. Found C, 69.89; H, 9.81; N,13.43.

3.1.2. Tris-{[2-(2-benzylhistamine)oxo]ethyl}amine (2). The starting trialdehyde **9** (0.2307 g, 0.50 mmol) was suspended in a 10 mL dry CH_3CN at rt followed by addition of solid histamine base **11** (0.1667 g, 1.50 mmol) in four portions while warming gently to dissolve all reactants. Stirring was continued at RT for 4 h after which rubber-like solid deposited at the flask bottom. This Schiff base was reduced in situ with excess $NaBH_4$ after adding 10 mL of MeOH. The compound was isolated as described above for **1** to obtain the title compound as a hygroscopic solid in 90% yield. (^{1}H NMR, $CDCl_3$, TMS, ppm) 2.74 (t, –NH*CH*$_2$CH$_2$Im, *J*=7.6 Hz), 2.83 (t, -NHCH$_2$*CH*$_2$Im, *J*=7.6 Hz), 2.95 (t, –N*CH*$_2$CH$_2$O, *J*=7.2 Hz), 3.75 (s, –Ar*CH*$_2$NH–), 3.99 (t, –NCH$_2$*CH*$_2$O, *J*=7.4 Hz), 6.63 (s, *Ar*), 6.83 (m, *Ar*), 7.17 (m, *Ar*), 7.34 (s, *Ar*). Anal calcd for $C_{42}H_{54}N_{10}O_3\cdot 2H_2O$: C, 64.43; H, 7.47; N, 17.89. Found C, 64.55; H, 7.59; N, 17.73.

3.1.3. Tris(2-aminoethyl-4-imidazolylmethyl) amine (3). Tris(2-aminoethyl)amine **12** (0.1462 g, 1.00 mmol) in 5 mL dry MeOH was added to a solution of 4-imidazolecarboxaldehyde **13** (0.2883 g, 3.00 mmol) in 5 mL of dry MeOH. The mixture was stirred at rt for 6 h followed by refluxing for 1 h. The resulting Schiff base was hydrogenated as described above with excess of $NaBH_4$. The workup procedure was the same as for **1** but the tripodal ligand was extracted many times with CH_2Cl_2. **3** was obtained as a hygroscopic solid in 68% yield. (^{1}H NMR, [D$_4$]MeOH, TMS, ppm) 2.89 (t, –N*CH*$_2$CH$_2$NH–, *J*=8.3 Hz), 3.24 (t, NCH$_2$*CH*$_2$NH–, *J*=8.5 Hz), 4.28 (s, Im*CH*$_2$NH–), 7.41 (s, *Ar*), 7.78 (s, *Ar*). Anal calcd for $C_{18}H_{30}N_{10}\cdot H_2O$: C, 53.45; H, 7.97; N, 34.63. Found C, 53.41; H, 8.05; N, 34.74.

3.2. NMR spectra

NMR spectra were taken at 400.13 MHz for 1-H with a Bruker AM 400 system, under conditions as given in the tables. Apparatus for kinetic analyses, see below.

3.3. Thermal melting curves

Thermal melting curves were obtained with a Cary 1 Bio UV–Vis spectrophotometer connected with a temperature-controller and interfaced to a PC. The melting curves were recorded as described in the literature[18] at different compound to nucleic acid phosphate ratios (*r*) by following the absorption change at 260 nm as a function of temperature with a heating rate of 0.5°C min^{-1}. T_m values were determined from the maximum of the first derivative, or tangentially from the graphs at the mid point of the transition curves. ΔT_m values were calculated by subtracting T_m of the free nucleic acid from T_m of the complex.

3.4. DNA cleavage experiments

Samples were incubated at 37°C for 2 h in 10 μL samples, as described earlier.[2k] The reactions were quenched by addition of 2 μL of a loading buffer containing 40 wt% saccharose, TRIS 0.89 M, boric acid 0.89 M, EDTA 1.0 M and a little bromophenolblue. The macrocyclic polyamines inhibit the migration of DNA in the gel electrophoresis; this was overcome by using ion exchange resin as previously reported.[2k] Electrophoresis was conducted on 0.9% agarose in a horizontal gel apparatus at 70 V for 2 h. The electrophoresis buffer contains 0.89 M TRIS, 0.89 M boric acid, 2 mM EDTA and 0.5 μg mL^{-1} ethidiumbromide. Quantification after electrophoresis was performed with an 'Eagle Eye II' densitometry system using the 'Zero-Dscan' software from Scanlytics; corrections for RF II portions present already in the starting material (about 10%) were applied.

3.5. BNPP cleavage experiments

The rate of *p*-nitrophenolate released was monitored[2l] at 400 nm (ε=6430 M^{-1} cm^{-1}) with a Cary 1 Bio UV–Vis spectrophotometer at 50°C. The required amount of BNPP solution was added to 1 mL of the reaction solution in quartz semi-microcuvettes of 1 cm path length. The reaction was monitored for a period of 1000 min. The rate constants were calculated from treatment of the data using a first order rate law.

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